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Changes in In vitro Brain and Spinal Cord Protein Phosphorylation After a Single Oral Administration of Tri-o-Cresyl Phosphate to Hens

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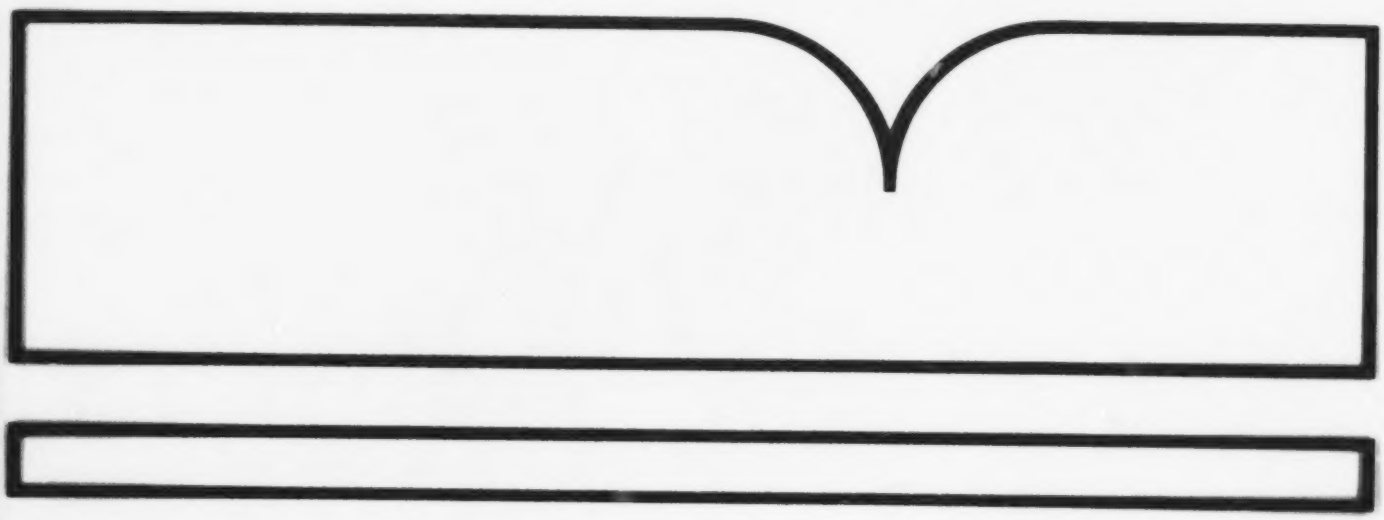

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16. ABSTRACT

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JOURNAL ARTICLE

Changes in In Vitro Brain and Spinal Cord Protein Phosphorylation After a Single Oral Administration of Tri-*o*-Cresyl Phosphate to Hens

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Abstract: The effect of a single oral 750 mg/kg dose of tri-*o*-cresyl phosphate (TOCP) on the endogenous phosphorylation of brain and spinal cord proteins was assessed in hens during the development of and recovery from delayed neurotoxicity. Crude membrane and cytosolic fractions were prepared from the brains and spinal cords of control and TOCP-treated hens at 1, 7, 14, 21, 35, and 55 days after treatment. Brain and spinal cord protein phosphorylation with [γ-^{32}P]ATP was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), autoradiography, and microdensitometry. TOCP administration conferred calcium and calmodulin dependence on the phosphorylation of a few brain cytosolic proteins and caused an increase in the phosphorylation of a number of other cytosolic and membrane proteins. This effect of TOCP was large in magnitude, and its time course reflected the onset of and recovery from the signs of ataxia and paralysis associated with delayed neurotoxicity in the hen. The molecular weights (M_r) and maximal phosphorylation (percent of control) for the most prominently affected bands were as follows: brain cytosol—50K (183%), 55K (575%), 60K (529%), 65K (273%), and 70K (548%); brain membranes—50K (622%) and 60K (697%); and spinal cord cytosol—20K (182%). The role of endogenous phosphorylation reactions in and their potential usefulness as biochemical indicators of delayed neurotoxicity are being explored further. **Key Words:** Protein phosphorylation—Tri-*o*-cresyl phosphate—Brain protein—Spinal cord protein. **Patton S. E. et al.** Changes in in vitro brain and spinal cord protein phosphorylation after a single oral administration of tri-*o*-cresyl phosphate to hens. *J. Neurochem.* **45**, 1567–1577 (1985).

Distinct from the acute cholinergic crisis resulting from their inhibition of acetylcholinesterase (AChE), many, but not all, organophosphorus esters (OPs) have also been shown to induce a delayed neurotoxicity, organophosphorus ester-induced delayed neurotoxicity (OPIDN). This condition is characterized by the dose-dependent development of ataxia and paralysis concurrent with a central-peripheral distal axonopathy, after a 6- to 14-day latent period following a single exposure (Abou-Donia, 1981). Internodal swelling and axonal degeneration, with subsequent Wallerian degeneration of the myelin, begin distally and progress somatofugally toward the terminals of large-diameter, long axons in the peripheral motor nerves and the spinal cord motor and sensory tracts (Bouldin and Cavanagh, 1979).

As many as 20,000 people were paralyzed in the late 1920s during Prohibition in the United States after drinking "Ginger Jake," a "tonic" that had become contaminated with 2% tri-*o*-cresyl phosphate (TOCP) (Smith et al., 1930). In 1958 another

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A preliminary account of this manuscript already has been presented (Patton et al., 1983*a*).

Abbreviations used: AChE, acetylcholinesterase; CaM, calmodulin; K, kilodaltons; MAP, microtubule-associated protein; M_r, electrophoretic mobility; OPs, organophosphorus esters; OPIDN, organophosphorus ester-induced delayed neurotoxicity; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TOCP, tri-*o*-cresyl phosphate.

10,000 people in Morocco experienced a similar delayed paralysis after cooking with oil adulterated with the turbojet lubricant and industrial solvent tritolyl phosphate, of which TOCP is the neurotoxically active isomer (Smith and Spalding, 1959). TOCP has become the prototype of a large class of organophosphates inducing a delayed neurotoxicity (OPIDN), the signs of which have been well-described (Abou-Donia, 1981). They are consistent, easily monitored, and uncomplicated by acute cholinesterase inhibition, when TOCP is the agent and the chicken the test animal of choice.

OPs are known to inhibit a variety of hydrolytic enzymes by covalent phosphorylation of serine residues in the active site. This mechanism of enzyme inhibition by OPs is most well-documented for AChE; however, plasma butyrylcholinesterase, serine proteases, and certain amidases are also inactivated through phosphorylation of serine residues (Eto, 1974). Although phosphorylation of these esterases has not been shown to be sufficient to cause the development of OPIDN, phosphorylation of serine residues with ATP as the phosphate donor has been shown to be a predominant mode of physiological posttranslational modification of proteins. Indeed, phosphorylation of several well-characterized neuronal proteins may play a pivotal role in synaptic function (for reviews, see DeLorenzo, 1981; 1982; Nestler and Greengard, 1983). Therefore, it seems plausible that organophosphorus esters, by analogy with AChE inhibition, may interfere with the functions of a crucial serine or other amino acid residues on neural enzymes or substrate proteins involved in endogenous phosphorylation, thereby adversely affecting the regulation of many neuronal processes. Indeed, we have found that administration of the organophosphorus compound TOCP to the chicken alters the endogenous phosphorylation of several brain cytosolic and membrane proteins (Patton et al., 1983*a*,*b*; Abou-Donia et al., 1984). In this study we have examined endogenous phosphorylation of brain and spinal cord proteins during the development, progression, and improvement from TOCP-induced delayed neurotoxicity.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP (4,500 Ci/mmol) was obtained from ICN (Irvine, CA, U.S.A.). Tris, ultrapure, was from Schwarz-Mann, Inc. (Spring Valley, NY, U.S.A.) and glycerol was from Mallinkrodt (Paris, KY, U.S.A.). All other materials used in the preparation of the polyacrylamide gels and associated buffers were of electrophoresis grade and were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.), as were Coomassie Brilliant Blue G-250 (protein assay dye reagent), plasma bovine γ-globulin, and Chelex 100. EGTA, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). TOCP (99%) was obtained from Eastman Kodak Co. (Rochester, NY, U.S.A.). Bovine brain calmodulin was provided by Dr. Thomas C. Vanaman, University of Kentucky Medical Center (Lexington, KY, U.S.A.).

Care and treatment of animals

Adult leghorn laying hens (*Gallus gallus* domesticus), each 14 months old and weighing approximately 1.7 kg, were obtained from Featherdown Farm (Raleigh, NC, U.S.A.). The time course was performed in two separate experiments: short term were killed at 1, 7, 14, or 21 days after treatment, and long term were killed at 35 or 55 days after treatment. Four groups of four hens each (short term) and two groups of seven hens each (long term) were given single oral doses of 750 mg/kg TOCP in gelatin capsules at the appointed time before being killed. The two control groups of four hens each were given empty gelatin capsules. After dosing, the birds were returned to their stainless steel cages in a humidity (40–60%) and temperature (21–23°C) controlled room with a 12-h light cycle and supplied with food and water ad libitum. To counter their reduced intake of food and water from bowls placed within their reach, paralyzed birds were given 10 ml of 1% Dyne High Calorie Liquid Diet and Dietary Supplement, Biolab Corp. (Norborne, MO, U.S.A.) and 20 ml of 10% sucrose by gavage daily. Body weights were monitored weekly, and the hens were examined daily for signs of neurological dysfunction, as described previously (Abou-Donia, 1978). At the termination of each experiment, the hens were anesthetized with CO_2 and killed by decapitation.

Preparation of subcellular fractions

Following sacrifice, brains and spinal cords (the latter expelled by injection of ice-cold 0.9% saline into the vertebral canal; Meikle and Martin, 1981) were removed immediately and immersed in ice-cold 0.32 *M* sucrose, 0.3 m*M* PMSF, 50 m*M* Tris-HCl (pH 7.4). The preparation of crude synaptosomal cytosolic and membrane fractions has been described previously (Patton et al., 1983*a*,*b*; 1985*a*,*b*).

Phosphorylation assays

The phosphorylation reaction was initiated by the addition of a 25 μl protein aliquot containing 100 μg of synaptosomal cytosol or 75 μg of crude P_2 membranes (Patton et al., 1985*a*,*b*) in 5.0 m*M* Tris (pH 8.0) to the reaction mixture. The standard assay also contained, in a final volume of 200 μl: 50 m*M* PIPES (pH 6.5), 10 m*M* $MgCl_2$, 0.3 m*M* PMSF, and 5.0 μ*M* [γ-^{32}P]ATP (4,500 Ci/mmol) at 35°C. Additionally, cytosolic proteins were incubated in the absence or presence of 50 or 100 μ*M* $CaCl_2$, or 100 μ*M* EGTA, whereas membrane proteins were incubated in the absence or presence of 300 μ*M* $CaCl_2$, alone or with 1 μg calmodulin (CaM). The reaction was terminated after 90 s for cytosol or 20 s for membranes, by the addition of 100 μl of an SDS-PAGE sample buffer [containing 0.125 *M* Tris-HCl (pH 6.8), 4.5% SDS, 20% glycerol, and 10% β-mercaptoethanol] followed by heating for 3 min in a 90°C water bath. Protein concentrations were determined by the method of Bradford (1976) using Coomassie Brilliant Blue G-250 with bovine plasma γ-globulin as a standard. The deionized water

used in the phosphorylation assays, as well as in the homogenization, lysis, and electrophoresis gel buffers was passed through a Chelex-100 column to ensure thorough removal of Ca^{2+} and other divalent cations. The assay was performed in plastic (polyethylene) test tubes, and all solutions in contact with the tissue samples before the addition of stop solution were stored in plastic vessels, to minimize vessel-related Ca^{2+} contamination.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), autoradiography, and microdensitometry

The synaptosomal cytosol and membrane proteins were resolved on 20-well SDS-polyacrylamide vertical slab gels (17 × 16 × 0.15 cm) (Bio-Rad Laboratories, Richmond, CA, U.S.A.), according to the procedure of Patton et al. (1985*a*,*b*). Electrophoresis was performed using the stacking, resolving, and running gel buffers described by Laemmli (1970) with 6% and 10% acrylamide in the stacking and resolving gels, respectively. Aliquots (50 μl) of the samples were subjected to electrophoresis under conditions of constant current (30 mA/gel through the stacking gel and 40 mA/gel through the resolving gel) at 13–15°C until the tracking dye reached the bottom of the gels (3.5–4.0 h). The apparent electrophoretic mobilities (M_rs) of the resolved proteins were determined from M_r standards (Bio-Rad Laboratories, Richmond, CA, U.S.A.) that had been subjected to electrophoresis under conditions identical to the tissue samples and are presented in kilodaltons (K). Following electrophoresis, the gels were fixed and stained for protein overnight with 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid. The gels were destained in 50% methanol and 10% acetic acid (2–3 h) in a diffusion destainer employing activated charcoal (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and then soaked for 1 h in 10% acetic acid and 1% glycerol (to prevent cracking) before drying for 2 h between two sheets of dialysis membrane under heat and reduced pressure (water aspirator). Autoradiographs of the ^{32}P-labelled proteins were obtained by placing the dried gels in close contact with Kodak X-OMAT RP x-ray film (0.5–3.0 days) at −70°C using intensifying screens (Dupont Cronex Lightning-Plus, CGR Corp., Raleigh, NC, U.S.A.; Laskey and Mills, 1977). The gel lanes and autoradiographs presented are representative of at least four to six separate tissue preparations. The amount of phosphoprotein in each autoradiographic band was quantified by determining the area under the corresponding peak in the densitometric scan obtained by using a Zeineh Soft Laser Densitometer (BioMed Instruments, Inc., Fullerton, CA, U.S.A.) interfaced with a PDP-11 computer (Digital Equipment Corp., Maynard, MA, U.S.A.) programmed for integration. Microdensitometry of the dried gels has previously failed to reveal a significant difference between control and (single dose, 750 mg/kg, day 21) TOCP-treated chickens in the Coomassie Blue staining of any protein band (Patton et al., 1983*b*).

Statistics

Statistical comparisons of protein phosphorylation between control and TOCP-treated chickens were based on the Student's unpaired two-tailed *t* test.

RESULTS

Variation in clinical signs with time after TOCP administration

Adult hens were treated orally with the organophosphorus compound TOCP at time zero. Development of delayed neurotoxicity was monitored at intervals up to 8 weeks after dosing. After 7 days, the TOCP-treated birds began to show signs of neurological dysfunction in the form of mild ataxia, characterized by diminished leg movement and reluctance to walk. Clinical signs progressed to disturbances in gait, unsteadiness, and frequency of falling on the floor. By day 14 most of the hens were unable to walk from a standing position. On day 21, they were completely paralyzed. However, they eventually regained their appetite and partially recovered their ability to walk by day 55, as they were force-fed a high-protein diet. The clinical condition ranged from mild to severe ataxia on day 55.

TOCP-treated hens suffered a progressive weight loss, which accelerated at the onset of paralysis (day 21), falling to a minimum of 63.2 ± 4.5% of their original weight at day 35, and only partially recovering thereafter to 84.3 ± 3.7% by day 55. Despite daily caloric and fluid supplements during paralysis, 57% of the 55-day hen group continued to lose weight and died before the end of the experiment. In contrast, the control group gained weight up to 118.8 ± 6.6% over the course of the experiment (Fig. 1).

Phosphorylation of brain proteins following the administration of TOCP

The possibility of delayed neurotoxicity-related biochemical alterations in the CNS endogenous phosphorylation by an organophosphate was investigated in the following way.

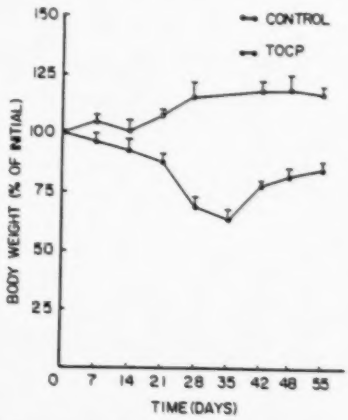

FIG. 1. Change in body weight of hens which survived a single oral 750 mg/kg dose of TOCP administered in gelatin capsules and control hens which received empty gelatin capsules over a 55-day observation period, expressed as a percentage of initial weight (mean ± SE, n = 3).

Synaptosomal cytosolic and crude P_2 membrane proteins obtained from the brains and spinal cords of control and TOCP-treated hens 1, 7, 14, 21, 35, and 55 days after treatment were phosphorylated with [γ-^{32}P]ATP in vitro by endogenous kinases. Subsequent separation by SDS-PAGE revealed the molecular weights of the phosphorylated substrate proteins. There was no variation between the samples from control and TOCP-treated animals in the relative abundance of any band in the Coomassie Blue protein staining pattern, regardless of time elapsed since dosing.

Brain soluble proteins. Differences in neural protein phosphorylation between control and TOCP-treated hens were analyzed qualitatively from the autoradiographic patterns of the dried gels (Figs. 2 and 3), and quantitatively, from the areas under the peaks in their densitometric scans. These area differences were expressed as percentages of the ^{32}P incorporated into proteins from control chickens under identical conditions (Table 1). The data show that TOCP ingestion resulted in significant enhancement of in vitro phosphorylation of several brain synaptosomal cytosolic proteins maximally 14–21 days after its administration. Phosphorylation in the presence of 50 μM Ca^{2+} of the prominent 55K and 68K brain cytosolic proteins increased progressively from 220.2% and 124.3% of control, respectively, at day 7 to 574.6% and 273.0% at day 14. It then decreased slightly to 514.9% and 176.7% at day 21, but was no longer significantly different from control by day 35. In addition to increasing the magnitude of the phosphorylation of these proteins, a 14-day wait after TOCP treatment apparently conferred sensitivity to exogenous Ca^{2+} upon the phosphorylation of several such proteins. Included in this category are the 55K band (574.6%, 50 μM Ca^{2+}), which was easily visible but much less Ca^{2+} sensitive in control than in treated hens, and two otherwise minor brain cytosolic protein bands, 60K (528.6%, 50 μM Ca^{2+}), and 72K (547.7%, 50 μM Ca^{2+}), which were barely visible in the autoradiographs of control hen samples, even when Ca^{2+} was added. To a considerably lesser extent, although significant at day 14, TOCP treatment increased the phosphorylation of the 20K band and the 68K band. The phosphorylation of the former was already Ca^{2+}-dependent in the controls (193.8%, 100 μM Ca^{2+}), while the phosphorylation of the 68K band (273.0%, 50 μM Ca^{2+}) never became more than scarcely Ca^{2+} sensitive.

Brain membrane proteins. Brain membrane proteins, obtained from the same animals as the brain cytosolic proteins, were analyzed by the same techniques (Figs. 4 and 5; Table 2). However, higher supplementary concentrations of Ca^{2+} and CaM were tested to differentially replenish essential cofactors lost with the soluble compartment upon fractionation. The time course of the TOCP-related increase in phosphorylation was similar for all the

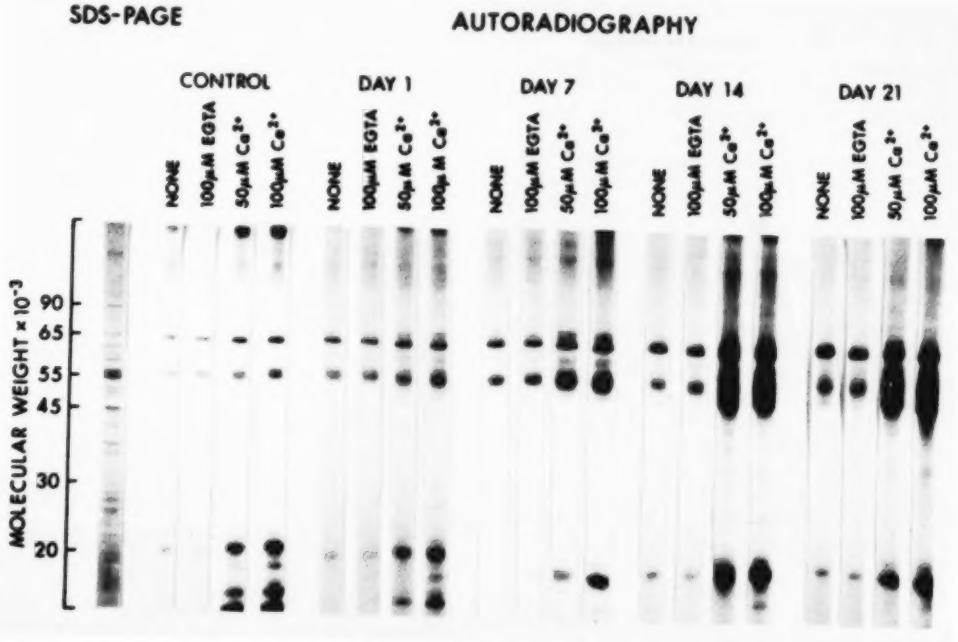

FIG. 2. Representative SDS-PAGE Coomassie Blue staining profile of hen brain synaptosomal cytosolic proteins, unchanged by indicated treatments, and autoradiographs of their in vitro ^{32}P incorporation under indicated conditions 0, 1, 7, 14, or 21 days after the hens received 750 mg/kg TOCP p.o.

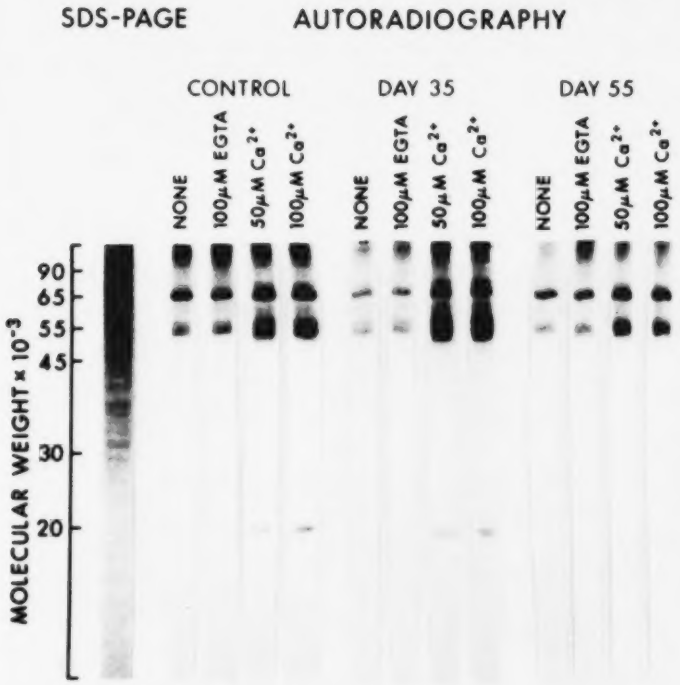

FIG. 3. Representative SDS-PAGE Coomassie Blue staining profile of hen brain synaptosomal cytosolic proteins, unchanged by indicated treatments, and autoradiographs of their in vitro ^{32}P incorporation under indicated conditions 0, 35, or 55 days after the hens received 750 mg/kg TOCP p.o.

brain membrane substrate proteins (20K, 50K, 60K, and 80K). Within 24 h of dosing, the potential of these brain membrane proteins to undergo phosphorylation was already detectably enhanced. It reached a maximal level at day 7, then was sustained or fell slightly until day 21, after which the only remaining enhancement that could be attributed to TOCP was in the 50K band and the 60K doublet (day 35). The phosphorylation of the 50K band, 60K doublet, and 80K band was stimulated by exogenous Ca^{2+}/CaM also regardless of TOCP treatment. However, it was enhanced the most in response to TOCP and maintained at a high level the longest in the absence of Ca^{2+} and CaM. This suggests the possible activation in response to TOCP of a membrane-bound protein kinase that does not require Ca^{2+} or CaM as cofactors. These proteins have similar electrophoretic mobilities to the Ca^{2+}-CaM-phosphorylated rat forebrain 62K and 48K proteins; the brainstem 49K protein; the cerebellar, brainstem, and spinal cord 58K proteins; and the widely distributed, neuron-specific 86K doublet Proteins Ia and Ib, whose distribution was described by Walass et al. (1983). The presence of Ca^{2+}, with or without CaM, made no difference to the phosphorylation of the 20K band, before or at any time after TOCP treatment. This band has been tentatively identified as myelin basic protein on the basis of its presence in purified myelin subfractions and absence from purified synaptosomal subfractions of similar preparations (S. E. Patton, D. M. Lapadula, and M. B. Abou-Donia, submitted).

Phosphorylation of spinal cord proteins following the administration of TOCP

Spinal cord soluble proteins. In the cytosolic fraction of spinal cords obtained from the same animals (Figs. 6 and 7; Table 3), enhancement of the Ca^{2+}-dependent phosphorylation of several bands of low electrophoretic mobility ($M_r \leq 20K$) was maximal at day 7 (20K, 18K, 14K) and declined somewhat at day 14 (20K, 18K, 14K) and day 21 (20K, 18K, 14K). This time course is similar to that observed in brain membranes but is enhanced earlier and lasts longer after TOCP than in brain cytosol, suggesting either an involvement in repair or a major role in the damage. No secondary rise during the recovery period was seen for this band in the brain cytosol. In contrast to the low M_r bands, phosphorylation of the four or more proteins in the 50–70K range in the spinal cord cytosol showed no noteworthy effect of TOCP until day 35, declining at day 55. This contrasts sharply with the dramatically increased Ca^{2+}-dependent phosphorylation of proteins of similar size in brain cytosol.

Spinal cord membrane proteins. TOCP intoxication had no significant effect on the phosphory-

TABLE 1. *Stimulation of brain cytosolic protein phosphorylation with time after TOCP treatment*

Ca^{2+} condition	M_r (K)	Protein phosphorylation (% of control)					
		Day 1	Day 7	Day 14	Day 21	Day 35	Day 55
None added	72	103.7	109.0	204.7[b]	137.3	53.5	199.7
	68	108.0	92.7	214.2	112.0	63.5	94.7
	60	103.3	90.5	142.9	106.1	70.2	152.5
	55	102.3	99.5	126.6	188.3	89.9	92.2
	20	97.2	80.7	110.4	110.3	29.9	90.7
100 μ*M* EGTA	72	110.1	99.5	182.7	268.7	187.9[a]	265.5[a]
	68	100.7	94.1	202.7[a]	99.1	83.4	111.9
	60	96.3	92.3	158.0	91.3	183.7	232.0
	55	118.4	113.2	160.7[a]	107.4	177.5	119.6
	20	109.7	73.2	124.0[a]	101.9	101.2	147.9
50 μ*M* Ca^{2+}	72	270.6	398.2	547.7[a]	575.5	337.6	484.3
	68	141.0[a]	124.3	273.0[c]	176.7[a]	103.6	124.5
	60	185.6	373.9	528.6[a]	556.0	428.8	488.5
	55	184.8	220.2[c]	574.9[c]	514.9[c]	735.4	703.4
	20	150.6	154.0	305.7	231.7	183.8	265.0
100 μ*M* Ca^{2+}	72	254.0	157.8	508.7[a]	601.2[a]	405.2	431.4
	68	121.8	116.1	255.9[b]	181.2	108.6	128.0
	60	207.9	161.2[b]	491.0[a]	523.0[b]	546.3	904.5
	55	199.5	190.6[b]	465.3[b]	468.6[c]	688.2	423.9
	20	118.3	91.9	193.8[a]	145.7	189.7	196.3

The net in vitro incorporation of ^{32}P from [γ-^{32}P]ATP into specific brain cytosolic proteins under standard assay conditions was quantified from the densitometric scans of the autoradiographs of the dried SDS-PAGE gels used to separate them. It is expressed as the percentage that the mean for each band at a given exogenous cofactor concentration on the indicated day after treatment with 750 mg/kg TOCP is of the mean under the same conditions for empty gelatin capsule control chickens (n = 4).

[a] Significantly different from control mean, $p < 0.05$; [b]significantly different from control mean, $p < 0.02$; [c]significantly different from control mean, $p < 0.005$.

lation of hen spinal cord membrane proteins (data not presented).

DISCUSSION

It has been suggested that the primary lesion in OPIDN results in a "chemical transection" of the distal axon, followed by axonal degeneration progressing toward the nerve terminal (Bouldin and Cavanagh, 1979). Swollen synaptic vesicles of the excitatory S type have been observed on the first day after onset of clinical signs in chickens (Bischoff, 1970). Then, about 14 days later, tangled filaments, disintegrating vesicles, and membranous material appear in the nerve endings, presumably at least partially transported there from the axon (Prineas, 1969). In the light of these histopathological findings, we investigated the time course of the effects of TOCP exposure on synaptosomal (nerve terminal) preparations. The clinical progression of ataxia to paralysis in treated chickens was used as an index of functional deficit for comparison with the biochemical alterations observed. Body weight and appetite were lost concurrently with the development of ataxia and paralysis. Weight and appetite were also regained in parallel with recovery of walking ability. Thus, the possibility of a contribution of nutritional deficiency to the development or maintenance of neurological dysfunction has not been ruled out.

We have employed in this study our previously determined optimal conditions of pH, temperature, incubation time, and protein concentration for in vitro protein phosphorylation with [γ-^{32}P]ATP in hen brain and spinal cord fractions (Patton et al., 1985*a*,*b*). The results show a dramatic increase was observed in the exogenous Ca^{2+} stimulation of in vitro ^{32}P incorporation into brain cytosolic proteins, particularly those with electrophoretic mobilities of approximately 55K, 60K, and 68K. The identities of these proteins and the reasons for their increased phosphorylation in response to TOCP are as yet unknown. Nevertheless, several interesting possibilities arise from consideration of other phosphoproteins described in the rat brain phosphorylation literature that are in this mobility range. These include synaptic vesicle Proteins IIIa (74K) and IIIb (55K) (Huang et al., 1982), the 55K regulatory subunit of type-II cyclic AMP-dependent protein kinase (Lohmann et al., 1980), the 50K and 60K Ca^{2+}-CaM-dependent phosphorylated synaptosomal cytosolic proteins observed by O'Callaghan et al. (1980*a*,*b*) with matching synaptic plasma membrane counterparts, and the autophosphorylatable 50K and 60K subunits of the Ca^{2+}-CaM-dependent kinase which also phosphorylates Synapsin

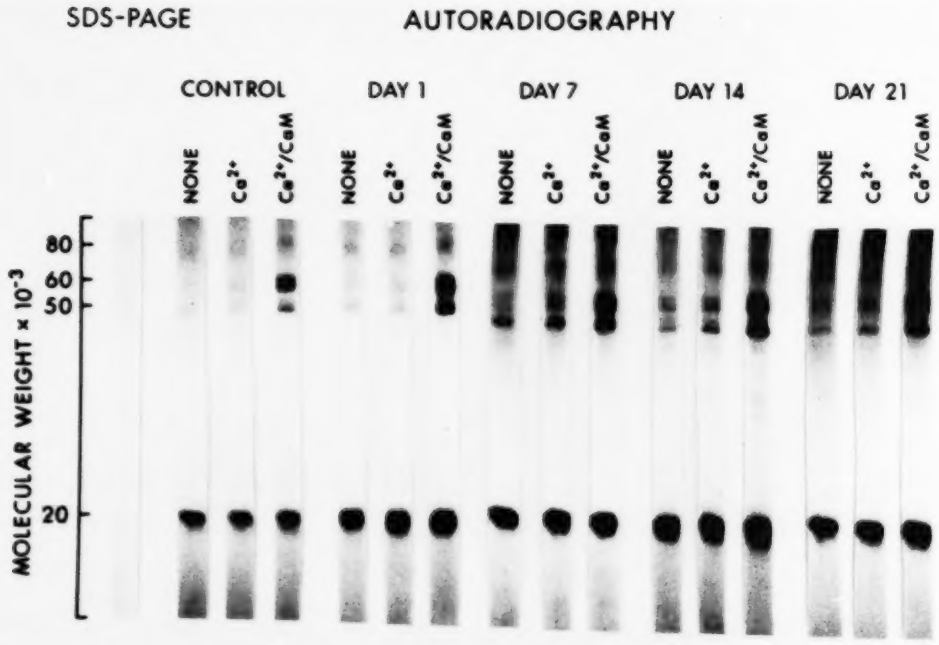

FIG. 4. Representative SDS-PAGE Coomassie Blue staining profile of hen brain membrane proteins, unchanged by indicated treatments, and autoradiographs of their in vitro ^{32}P incorporation under indicated conditions 0, 1, 7, 14, or 21 days after the hens received 750 mg/kg TOCP p.o.

I (Kennedy, 1983), microtubule associated protein MAP-2, and tubulin (Julien and Mushynski, 1981; Goldenring et al., 1983).

The following observations suggest that TOCP-induced Ca^{2+}-CaM-dependent protein phosphorylation resulted from increased autophosphorylat-

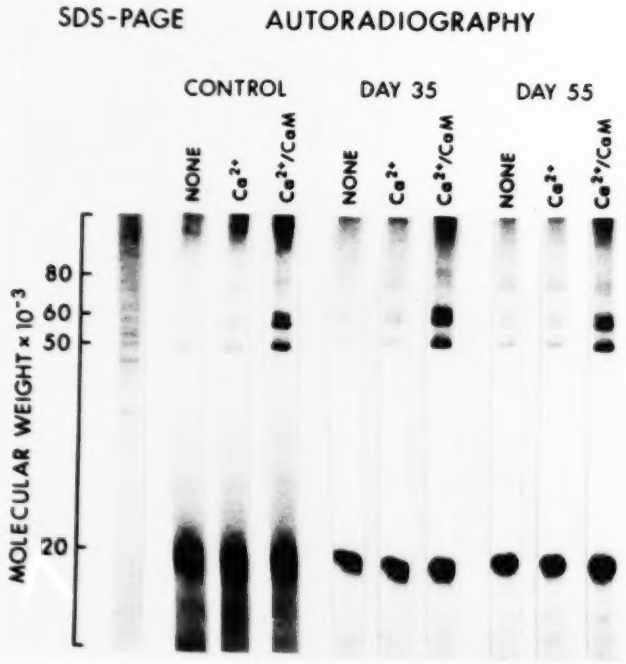

FIG. 5. Representative SDS-PAGE Coomassie Blue staining profile of hen brain membrane proteins, unchanged by indicated treatments, and autoradiographs of their in vitro ^{32}P incorporation under indicated conditions 0, 35, or 55 days after the hens received 750 mg/kg TOCP p.o.

TABLE 2. *Stimulation of brain membrane protein phosphorylation with time after TOCP treatment*

Cofactor condition	M_r (K)	Protein phosphorylation (% of control)					
		Day 1	Day 7	Day 14	Day 21	Day 35	Day 55
None added	80	309.4[b]	461.0[b]	314.3[c]	345.1[c]	74.8	89.6
	60	555.6[a]	669.7[b]	696.9[c]	684.7[c]	119.0	154.1
	50	394.2[b]	622.5[a]	486.7[c]	276.5[b]	91.6	132.4
	20	310.3[b]	383.0[b]	296.7	200.2	77.2	96.9
300 μM Ca^{2+}	80	195.4	258.3	138.2	182.1	105.1	107.6
	60	295.7	340.2[a]	287.8	276.4[a]	160.5[a]	149.2
	50	276.7[a]	365.3[a]	296.0[b]	184.2	126.4	138.5
	20	337.4[c]	379.2[c]	258.6	170.7	90.8	92.4
300 μM Ca^{2+} Calmodulin	80	170.7	229.1	164.9	404.5	132.3	100.4
	60	230.1	261.1	207.5	169.7	156.2[b]	132.3
	50	303.4	359.8	423.4[a]	336.1[b]	170.6[a]	159.4
	20	339.8[b]	393.6[a]	286.2	214.4	131.4	142.6

The net in vitro incorporation of ^{32}P from [γ-^{32}P]ATP into specific brain membrane proteins under standard assay conditions was quantified from the densitometric scans of the autoradiographs of the dried SDS-PAGE gels used to separate them. It is expressed as the percentage that the mean for each band at a given exogenous cofactor combination, on the indicated day after treatment with 750 mg/kg TOCP, is of the mean under the same conditions for empty gelatin capsule control chickens (n = 4).

[a] Significantly different from control mean, $p < 0.05$; [b]significantly different from control mean, $p < 0.02$; [c]significantly different from control mean, $p < 0.005$.

able 50K protein kinase. (a) The increase in phosphorylation of the three major brain cytosolic bands (20K, 55K, and 68K) is much more striking in the presence of Ca^{2+} than in its absence. (b) At least three new substrates for Ca^{2+}-dependent phosphorylation, with apparent molecular weights of 50K, 60K, and 70K, suggest possible kinase activation, particularly when the first (50K) is known to have the same M_r as an autophosphorylatable subunit of a Ca^{2+}-dependent tubulin kinase which satisfies all the criteria (Kennedy, 1983). Of course, an increase in soluble proteins available for phos-

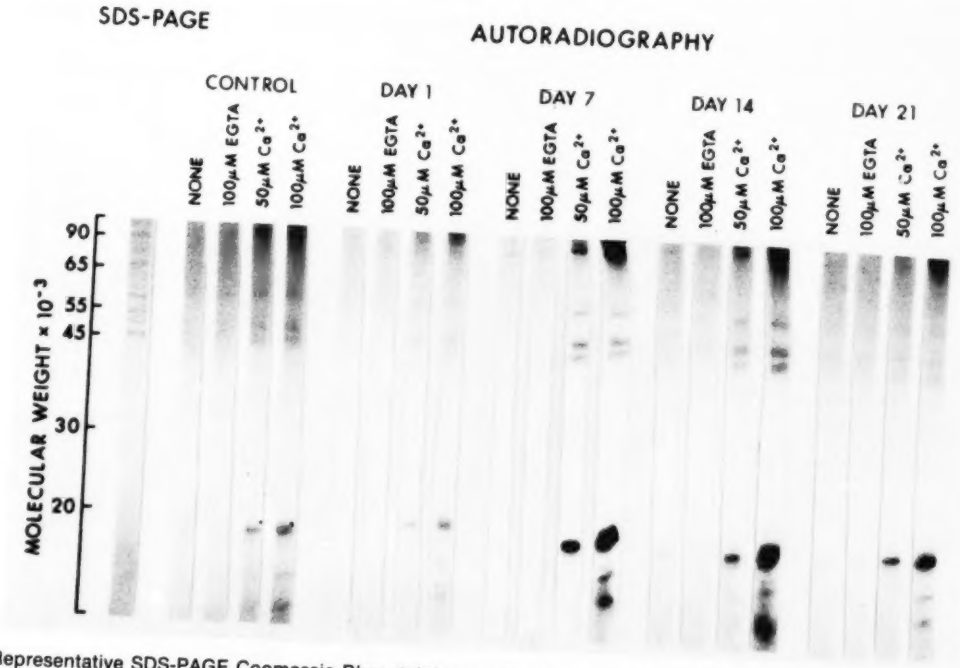

FIG. 6. Representative SDS-PAGE Coomassie Blue staining profile of hen spinal cord cytosolic proteins, unchanged by indicated treatments, and autoradiographs of their in vitro ^{32}P incorporation under indicated conditions 0, 1, 7, 14, or 21 days after the hens received 750 mg/kg TOCP p.o.

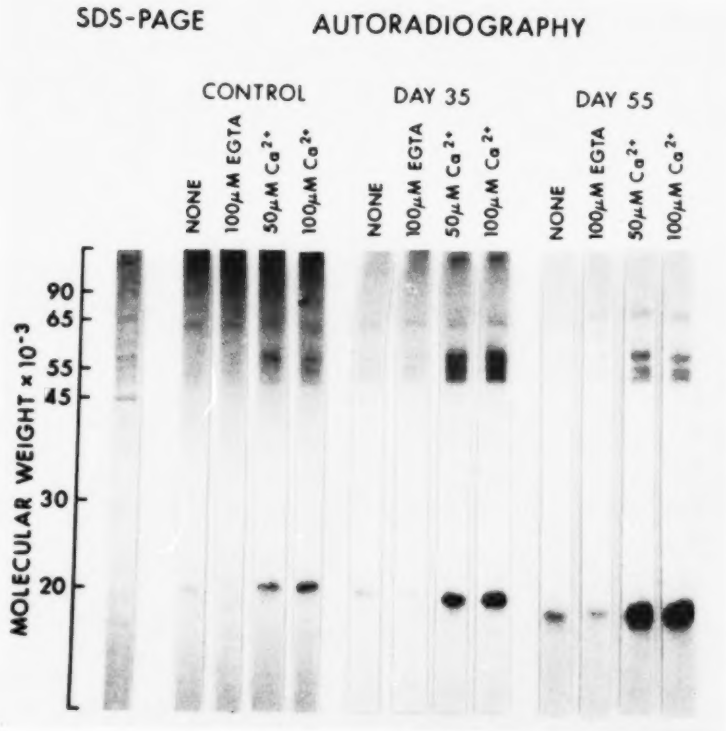

FIG. 7. Representative SDS-PAGE Coomassie Blue staining profile of hen spinal cord cytosolic proteins, unchanged by indicated treatments, and autoradiographs of their in vitro ^{32}P incorporation under indicated conditions 0, 35, or 55 days after the hens received 750 mg/kg TOCP p.o.

phorylation might only indicate breakdown of cellular structures.

The 55K doublet from the brains of hens paralyzed by TOCP, which is so highly phosphorylated in the presence of Ca^{2+}, does comigrate on one-dimensional SDS-PAGE with purified bovine- and β-tubulin (provided by Dr. Richard D. Irwin). DeLorenzo and co-workers (DeLorenzo and Freedman, 1977; DeLorenzo, 1980; DeLorenzo et al., 1982; Burke and Delorenzo, 1981, 1982*a*,*b*) have observed the formation of twisted filamentous polymers consequent to the endogenous Ca^{2+}- and CaM-dependent phosphorylation of rat brain synaptoplasmic tubulin. These polymers are distinct from either microtubules or tubulin ribbons or sheets. If our in vitro findings reflect an increased Ca^{2+}- and CaM-dependent phosphorylation of neuronal tubulin in vivo after exposure to TOCP, then it is possible that abnormally twisted phosphotubulin filaments are involved in OPIDN. They may be related to the tangled filaments noticed by Prineas (1969) in nerve endings 14 days after onset of this syndrome.

The proteins with $M_r \leq 20K$ in the brain and spinal cord soluble fractions were phosphorylated in a Ca^{2+}-dependent manner even before TOCP treatment. Proteins of similar size in the rat brain soluble fraction have been reported to be phosphorylated by a kinase requiring both Ca^{2+} and phosphatidlyserine as cofactors (Walaas et al., 1983). Perhaps inclusion of the brainstem in our preparation afforded sufficient liberation of phospholipids acceptable as cofactors for the relevant kinase.

The time course of in vitro protein phosphorylation effects after TOCP treatment was studied in spinal cord and brain fractions, since there is pathological and clinical evidence of more damage to the spinal cord than to the brain by this chemical (Abou-Donia, 1981). Consequently, fractions prepared in the same way from both tissues were compared.

The four or more proteins in the 50–70K range in the spinal cord cytosol showed no noteworthy effect of TOCP until day 35, declining at day 55. This contrasts sharply with the dramatically increased Ca^{2+}-dependent phosphorylation of proteins of similar size in brain cytosol. On the other hand, phosphorylation of the 20K band in spinal cord cytosol lasts longer after TOCP than in brain cytosol, suggesting an involvement in repair mechanisms. No secondary rise during the recovery period was seen for this band in the brain cytosol.

In conclusion, the effect of TOCP intoxication on in vitro phosphorylation of brain and spinal cord synaptosomal proteins appears to follow the time course described by Prineas (1969) for the morphological appearance of neuronal breakdown products

TABLE 3. *Stimulation of spinal cord cytosolic protein phosphorylation with time after TOCP treatment*

Ca^{2+} condition	M_r (K)	Protein phosphorylation (% of control)					
		Day 1	Day 7	Day 14	Day 21	Day 35	Day 55
None added	60	109.9	110.3	86.8	81.7	134.7	94.8
	55	134.7	145.3	105.4	99.8	172.4[a]	162.6
	20	224.0	172.8	169.9	133.0	158.5	168.1
	18	239.9	210.7[b]	173.7	129.0	—	—
	14	188.0	173.0	140.7	100.8	—	—
	10	135.0	105.0	54.4	108.7	—	—
100 μ*M* EGTA	60	115.5	88.9	81.0	65.9	119.2	113.2
	55	91.4	107.4	84.6	70.5	149.7	144.4
	20	218.6	182.0[a]	147.3	161.9	149.9	165.2
	18	236.1	169.9[a]	135.9	131.7	—	—
	14	196.2	227.5[a]	161.1	136.1	—	—
	10	128.1	190.6	54.0[a]	139.0	—	—
50 μ*M* Ca^{2+}	60	192.0[c]	157.2	126.9	100.0	282.8	155.1
	55	122.1	143.6	117.9	103.8	362.5[a]	224.2
	20	147.8	148.8	146.8	117.6	182.7	188.2
	18	117.1	148.9[b]	137.6	109.7	—	—
	14	109.9	139.7	82.8	71.6	—	—
	10	88.0	145.1	97.1	83.1	—	—
100 μ*M* Ca^{2+}	60	92.7	104.4	100.3	74.4	282.4	143.6
	55	65.8	85.1	75.3	60.7	350.9[a]	213.7
	20	118.6	126.0	127.0	92.4	160.8	174.0
	18	107.5	114.2	104.6	81.5	—	—
	14	100.7	91.3	86.8	72.1	—	—
	10	120.9	223.8[a]	214.0	129.8	—	—

The net in vitro incorporation of ^{32}P from [γ-^{32}P]ATP into specific spinal cord cytosolic proteins under standard assay conditions was quantified from the densitometric scans of the autoradiographs of the dried SDS-PAGE gels used to separate them. It is expressed as the percentage that the mean for each band at a given exogenous cofactor concentration on the indicated day after treatment with 750 mg/kg TOCP is of the mean under the same conditions for empty gelatin capsule control chickens (n = 4).

[a] Significantly different from control mean, $p < 0.05$; [b]significantly different from control mean, $p < 0.02$; [c]significantly different from control mean, $p < 0.005$.

in the nerve endings. It also correlates with the onset of and recovery from the clinical signs of ataxia and paralysis associated with delayed neurotoxicity in the hen. The delayed onset of this phosphorylation effect, in contrast to immediate, brief "neurotoxic esterase" inhibition (Johnson, 1981, 1982), ties it much more closely to the morphological and clinical manifestations of the syndrome in subsequent events. Mechanisms for increasing protein phosphorylation to be considered in future studies include protein kinase activation, phosphoprotein phosphatase inhibition, ATPase inhibition, and liberation of new substrate proteins from damaged axonal membranes and cytoskeleton. It remains to be determined whether the enhanced protein phosphorylation capacity observed during OPIDN results from covalent organophosphorylation of neural proteins.

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